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Changes in Cellular Retinoic Acid Binding Proteins and Nuclear Retinoic Acid Receptors in Murine Melanoma Cell Lines Following Retinoic Acid Exposure

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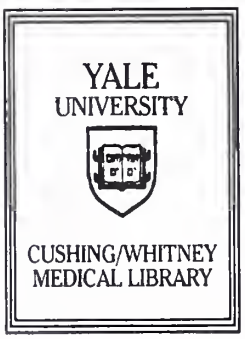
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Changes in Cellular Retinoic Acid Binding Proteins and Nuclear Retinoic Acid Receptors In Murine Melanoma Cell Lines Following Retinoic Acid Exposure.

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by
Raburn M. Mallory
1997

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CHANGES IN CELLULAR RETINOIC ACID BINDING PROTEINS AND NUCLEAR RETINOIC ACID RECEPTORS IN MURINE MELANOMA CELL LINES FOLL0WING RETINOIC ACID EXPOSURE. Raburn M. Mallory and Laura M. Roman. Department of Cellular and Molecular Physiology, Yale University, School of Medicine, New Haven, CT.

Retinoic acid (RA), a Vitamin A metabolite, has been shown to inhibit carcinogenesis in a variety of animal models and suppress *in vitro* transformation of certain cell lines. Growth inhibition by RA is associated with a commitment to differentiation induced by an altered pattern of gene expression. Evidence exists that two classes of proteins mediate these effects: (1) the nuclear RA receptors (RARs and RXRs), members of the steroid/thyroid superfamily of ligand-activated transcription factors, which modulate the expression of RA-responsive genes and (2), cellular retinoic acid binding proteins (CRABPI and CRABPII), which are found in many RA-responsive embryonic tissues and tumor cells. We hypothesize that these proteins sequester RA in the cytoplasm limiting its availability to the nuclear receptors.

Our studies focused on defining the roles of these proteins and the tissue plasminogen activator (tPA) system in tumor metastasis. Using two sublines of murine melanoma that differ in their metastatic potential (B16 F1 low potential and B16 F10 high potential) we have shown a clear dose-dependent inhibition of proliferation following RA treatment (10^{-5}M to 10^{-7}M), with F1 cells demonstrating greater sensitivity to RA's effects. Both cell lines show induction of RAR β mRNA after RA exposure, with higher levels of transcript noted in F10 cells. Changes in RAR α were not seen. Administration of RA also resulted in a dose-dependent increase in CRABPII mRNA in F10 cells. In contrast high concentrations (10^{-5} M) of RA were required for induction in F1 cells. These results were corroborated by western analysis. CRABPI mRNA and protein were not detected either before or after RA incubation. RA administration resulted in a dose-dependent increase in tPA secretion, with no differences between cell lines apparent.

RA treatment of murine melanoma cell lines induces clear changes in proliferation, tPA secretion and the levels of RA binding proteins. Increased levels of CRABPII in F10 cells may confer resistance to RA treatment.

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LIST OF ABBREVIATIONS

BM	basement membrane
cDNA	complementary deoxyribonucleic acid
CRABPI	cellular retinoic acid binding protein I
CRABPII	cellular retinoic acid binding protein II
CRBPI	cellular retinol binding protein I
DEPC	dierthylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECM	Extracellular matrix
EDTA	ethylenediaminetetra-acetate
FBS	fetal bovine serum
g	gravity
IFN	interferon
IU	international units
M	molar
ml	mililiter
mM	milimolar
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
Nacl	sodium chloride
NaOac	sodium acetate
nm	nanometer
PA	plasminogen avtivator
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RNA	ribonucleic acid
RXR	retinoid X receptor
SD	standard deviation

SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
tPA	tissue-type plasminogen activator
TPA	12-0-tetradecanoylphorbol-13-acetate
ug	microgram
ul	microliter
uPA	urokinase-type plasminogen activator

INTRODUCTION

OVERVIEW

Vitamin A and its metabolites, which are termed retinoids, play an essential role in many biologic processes. The function of Vitamin A in the visual cycle has been known since 1968 (Wald, 1968), and its importance in reproduction, fetal development and the regulation of both cell proliferation and differentiation is now being elucidated. Studies have demonstrated that one of its metabolites, all-*trans* retinoic acid (RA), has the potential to suppress the malignant transformation of many tumor cell lines *in vitro* (Harisiadis et al., 1978) and inhibit carcinogenesis *in vivo* . Retinoic acid is also showing some clinical promise in the treatment of promyelocytic leukemia (Frankel et al., 1992) and premalignant lesions of the head and neck (Hong et al., 1990). This introduction examines several areas of retinoid biology, specifically: (1) The role of retinoids in cancer chemoprevention and tumor invasion and (2) the proteins through which RA exerts its effects - the nuclear retinoic acid receptors (RARs and RXRs) and the cellular retinoic acid-binding proteins (CRABPs).

CANCER CHEMOPREVETION

Vitamin A (retinol) is derived from the ingestion and metabolism of naturally occurring retinyl esters of carotenoids. The latter compounds serve not only as pro-vitamins but also function as anti-oxidants. Epidemiological studies show a clear inverse relationship between the ingestion of carotenoids and lung cancer (Kvale et al., 1983) and (Shekelle et al., 1981). The role of carotenoid and retinoid intake in other cancers is less well defined. A modest protective effect is reported for breast and colon cancer, but a positive association between Vitamin A intake and prostate cancer has also been noted (for

review and references see Hunter in (Blomhoff, 1994)). The *in vitro* evidence that retinoids can prevent malignant transformation is overwhelming and the subject of a recent review (Lotan, 1996). A summary of significant findings is presented here. Retinoids are documented to inhibit transformation of cultured mouse embryo cells exposed to γ irradiation, and all-*trans*-RA provided similar protection to cells exposed initially to 3-methylcholanthrene followed by 12-0-tetradecanoylphorbol-13-acetate (TPA). Animal data provides further support for the efficacy of retinoids in cancer chemoprevention. The two-stage mouse skin carcinogenesis model is particularly well studied. Topical retinoids administered concurrently with TPA decreased the formation of papillomas and carcinomas in this model, while dietary all-*trans*-RA inhibited mouse skin tumor promotion by a number of different agents. Similar anti-carcinogenic effects were seen in many other organ systems and species. For example, rats surgically treated for the development of a mammary tumor after exposure to carcinogens developed fewer new tumors when retinoids were added to their diet. Human studies of retinoids have focused mainly on individuals known to be at higher risk for developing cancer. This group includes individuals with premalignant lesions as well as those who have had a primary tumor treated but remain at risk for developing a second primary. Regression of premalignant lesions was obtained when patients with actinic keratoses, dysplastic nevi or cervical intraepithelial neoplasia were given retinoids. In addition, clinical trials involving both breast cancer patients and patients with non-small cell lung cancer report a decreased incidence of second primary tumors with retinoid treatment.

The anti-carcinogenic effects of retinoids are generally thought to occur at the stage of tumor promotion where initiated cells proliferate to form pre-neoplastic lesions and then convert to malignancy. Promotion may be inhibited by retinoids in a number of ways: Retinoids are known to have anti-proliferative effects (Lotan, 1980); to enhance cell differentiation (Sani et al., 1984), and to decrease tumor cell invasion *in vitro* (Situ et al., 1993). The role of RA in cell differentiation has been extensively studied and is beyond the

scope of this introduction. The interactions between RA and cell proliferation and invasion will be outlined in more detail.

CELL PROLIFERATION

The rationale for the use of RA as a chemopreventive agent is based to an extent on its ability to inhibit growth (Lotan, 1980). Numerous cell lines including mammary adenocarcinomas, lymphomas, osteosarcomas, myelomas and melanomas are susceptible to the effects of RA. This inhibition is not based on the growth rate of the cells and is not consistently seen, as some cell lines are actually stimulated to grow in the presence of RA. There is conflicting data regarding the mechanism of RA induced growth inhibition. Accumulation of cells in the G1 phase of the cell cycle was initially proposed (Vitale et al., 1986), however, recent evidence clearly implicates induction of apoptosis in this process. P19 embryonal carcinoma cells treated with retinoic acid A show the characteristic DNA fragmentation of apoptosis by propidium iodide staining (Horn et al., 1996) and similar results are noted in HL-60 cells (Nagy et al., 1995), F9 cells (Atencia et al., 1994) and cervical carcinoma cell lines (Oridate et al., 1995). A role for RAR β mediated inhibition of proliferation is proposed by Liu et al., (1996). These authors introduced a RAR β expression vector into breast cancer cell lines resistant to the effects of RA. This resulted in the acquisition of sensitivity to the growth-inhibitory effects of RA and in increased apoptosis. RAR α (Zhang et al., 1995), the RXRs (Nagy et al., 1995) and CRABPII (Delva et al., 1993) are also proposed to mediate the anti-proliferative effects of RA.

TUMOR INVASION

Invasion is considered the hallmark of malignant tumors (reviewed by Mignatti and Rifkin, (1993)). As cells metastasize to different sites in the body they must initially disrupt a normally impermeable basement membrane (BM), migrate through the interstitial stroma, enter the vascular system and finally extravasate to form secondary tumors.

Pathologically, the switch from a benign to a malignant phenotype is determined once cells have completed the initial step and the BM is no longer intact. The BM is composed of glycoproteins (including laminin and fibronectin), glycosaminoglycans, proteoglycans and multiple forms of collagen. Disruption of this surface by invading tumor cells requires the local expression of numerous degradative enzymes. These enzymes are generally divided into three major groups; 1) the serine proteases 2) the matrix metalloproteinases and 3) the cysteine proteases. The serine proteases include tissue type-plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). These enzymes are secreted in latent form and once activated convert plasminogen to plasmin. Plasmin is a protease with broad degradative abilities, able to digest both laminin and fibronectin as well as the protein core of proteoglycans. The matrix metalloproteinases (MMPs) are similarly secreted as pro-enzymes and upon activation degrade glycoproteins, elastin and the core of proteoglycans. The cysteine proteinases are characterized by Cathepsin B, a primarily lysosomal protein optimally active at acidic pHs. Known substrates for this enzyme include collagen and pro-uPA. Although this enzyme is normally restricted intracellularly, human breast carcinoma cell lines have been shown to secrete it into conditioned media, possibly implicating it in BM degradation (Poole et al., 1978).

While expression of all three classes of enzymes may be required for successful degradation of the BM, it has been argued that a proteinase cascade triggered by plasminogen activation plays a key role in tumor cell invasion (Mignatti, 1986). This contention is based on three main lines of evidence. First, plasminogen is fairly ubiquitous in plasma and tissue, and the generation of even small amounts of plasminogen activators (PAs) results in the formation of large amounts of plasmin. This occurs as plasmin, once formed, converts pro-PAs to PAs thus increasing its own production through a positive feedback mechanism. Secondly both plasminogen and uPA receptors are colocalized on cell membranes. This colocalization not only vastly increases the amount of plasmin formed, but also protects it from anti-plasmin, the serum inhibitor of plasmin

action (Meissauer et al., 1992). Lastly plasmin in cell culture has demonstrated the ability to activate latent MMPs and elastase. These enzymes may act in a sequential fashion with plasmin in the degradation of BM architecture (Montgomery et al., 1993) and (Mueller, 1996). The central role of plasmin in the degradation of the BM is summarized in Figure 1.

The role that plasminogen activators play in metastasis has been extensively studied. For reviews see Dano et al., (1985) and Saksela and Rifkin, (1988). Wang and Mannick noted in 1980 that B16-F10 murine melanoma cells had significantly higher fibrinolytic activity than B16-F1 cells, which correlated to their reported *in vivo* metastatic potential (Wang et al., 1980). The relative contributions of tPA and uPA to invasion are not completely clear. It has been suggested that only uPA expression correlates with metastatic ability in B16 murine melanoma cell lines (Huijzer et al., 1995). These authors note that B16-F1 cells had higher levels of tPA activity than more metastatic B16-F10 cells, and that uPA secretion appeared to reflect the metastatic characteristics of these tumor sublines. Further, the expression of high levels of tPA may actually be a positive prognostic marker for survival in breast cancer patients (Duffy et al., 1988). However, NIH 3T3 cells transfected with tPA were able to cross reconstituted extracellular matrices (ECMs) more efficiently than control cells (Axelrod et al., 1989) and the human colon carcinoma cell line Col 115 shows a correlation between *in vitro* invasion and tPA activity (Cajot et al., 1986).

The effect of RA on tPA expression appears to be cell line specific. Subtypes of the human A375 melanoma cell line express PAs primarily in the form of tPA, with the most metastatic subtype (A375P-5) showing the highest levels. Treatment of this cell line and the other A375 derived cell lines with 10^{-6} M RA for ten days resulted in a decrease in tPA expression and *in vitro* invasion (Hendrix et al., 1990). HT1080 fibrosarcoma cells and human umbilical vein endothelial cells on the other hand, respond to RA treatment by increasing tPA secretion (Bulens et al., 1995) and (Lansink and Kooistra, 1996). Despite these inconsistencies, the recent discovery of a RARE in the human tPA promoter (Bulens

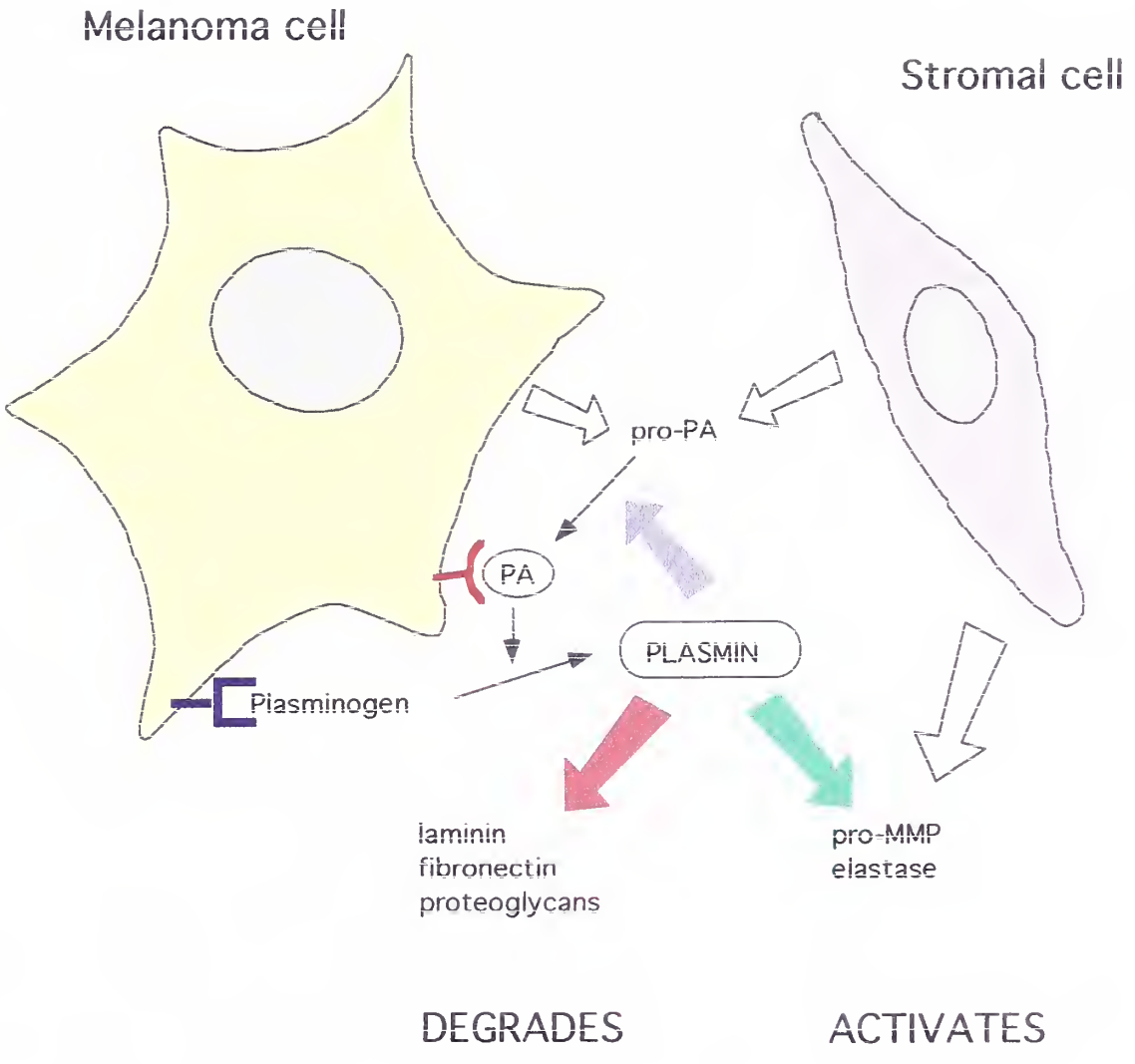

Figure 1. Cartoon depicting plasmin activation and its role in tumor cell invasion. PA = Plasmin activators. MMP = matrix metalloproteinase

et al., 1995) provides the mechanism by which RA can directly alter the expression of this gene.

METASTATIC MELANOMA

Murine and human melanoma cell lines are often used in studies of tumor cell invasion. These cells are used not only for their extreme invasiveness but also for their clinical relevance. Melanoma is now the most rapidly increasing cancer in white populations with a 1.1% lifetime prevalence in the US (Armstrong and Kricker, 1994). One of the most invasive cancers known, melanoma is particularly insidious because of the rapid dissemination of tumor cells throughout the body. Retinoic acid has been considered as a possible therapy for melanoma for several reasons: (1) Both melanocytes and melanoma cell lines show marked reduction in growth after administration of RA (Lotan, 1980) and (Fligiel et al., 1992): (2) RA has demonstrated efficacy in reducing tumor cell invasion both *in vitro* (Hendrix et al., 1990) and in *vivo* (Edward et al., 1992).

NUCLEAR RA RECEPTORS

The mechanism by which RA exerts its effects has become somewhat clearer since the discovery of a nuclear retinoid receptor in 1987. The nuclear retinoid receptors are members of the steroid/thyroid/Vitamin D_3 superfamily of ligand activated transcription factors. Two families of these receptors exist, the RA receptors (RARs) and the retinoid X receptors (RXRs).

The RARs were the first family to be cloned and consist of four subtypes: RAR α, RAR β, RAR γ and RAR δ. The first three receptors were isolated from both human (Petkovich et al., 1987),(Brand et al., 1988) and (Krust et al., 1989) and mouse cell lines (Zelent et al., 1989) while RAR δ remains restricted to newt tissue (Ragsdale et al., 1989). Each of these subtypes is able to generate at least two different isoforms through alternative splicing or use of different promoters (Chambon, 1996). The RARs bind all-*trans*-retinoic

acid and, with lower affinity, 9-*cis*-retinoic acid (9-*cis*-RA). The nucleotide homology between subtypes across species is high and specific patterns of expression are seen in mouse embryogenesis providing evidence that each RAR has distinct functions in different tissues.

The expression pattern and response to RA of the RARs have been investigated in a number of tissues and malignant cell lines. RAR α appears to be constitutively expressed in all adult mouse tissue (Zelent et al., 1989), in both S91-C2 and B16 murine melanoma cell lines (Clifford et al., 1990) and (Niles, 1995), as well as in several head and neck squamous cell carcinoma cell lines (Zou et al., 1994). Treatment of these tissues with RA resulted in either no change in RAR α levels, or at most a modest two-fold increase. In contrast RAR β was not detected in either of the two murine melanoma cell lines, seen mainly in muscle and brain tissue from the adult mouse and, was differentially expressed by the squamous cell carcinoma lines. Notable increases in RAR β expression were consistently seen in all tissues following incubation with RA and inductions greater than five-fold were common. In the adult mouse RAR γ is largely restricted to the skin, and transcript levels are diminished 50% following RA treatment (Zelent et al., 1989). RAR γ was detected in all of the malignant cells with very modest to no changes reported to follow RA exposure (Zou et al., 1994).

The second class of nuclear receptors, the RXRs, also has three main subtypes (Mangelsdorf et al., 1992). In structure the RXRs resemble the RARs but significant differences are present in the ligand binding domains. These receptors specifically bind and are activated 9-*cis*-RA (Heyman et al., 1992). The exact mechanism by which RA alters gene expression has recently been clarified. Each RXR isoform appears to have the ability to form heterodimeric complexes with the Vitamin D_3 receptor, the thyroid hormone receptor and the RARs. These interactions result in a significant increase in the binding affinities for the respective response elements (Yu et al., 1991). The retinoic acid response element (RARE) consists of a direct repeat of the hexanucleotide AGGTCA. It has been

shown that the number of nucleotides that separate this repeat help determine binding specificity (Umesono et al., 1991). RAR-RXR heterodimers are able to activate elements separated by 1, 2 and 5 nucleotides. Durand et al., (1992) note that while each member is able to activate transcription independently, the relative contributions appear to be dependent on the specific RARE and may also vary according to the concentration of retinoid ligand present. Differential gene activation may also reflect modifications in the ligand-binding, or activation domains of the nuclear receptors. Genes known to have functional RAREs include RAR α_2, RAR β_2, murine laminin B1, CRABPII and cellular retinol binding protein I (CRBPI).

CELLULAR RETINOIC ACID-BINDING PROTEINS

A second group of proteins, the cellular retinoic acid-binding proteins (CRABPI and CRABPII) is known to mediate the effects of RA. The cDNAs for CRABPI and CRABPII have been cloned from various species (Shubeita et al., 1987), (Stoner and Gudas, 1989), (Giguere et al., 1990) and (Astrom et al., 1991). These proteins belong to the family of cellular lipid binding proteins which include the myelin protein P2, numerous fatty acid-binding proteins and the cellular retinol binding proteins. As a class these proteins are known to bind, transport and metabolize low molecular weight hydrophobic substances (Blomhoff et al., 1990).

Murine CRABPI and CRABPII are small proteins of 136 amino acids, and are evolutionarily highly conserved. CRABPI appears somewhat more conserved as the human and mouse forms share 99.3% identity, however, human and mouse CRABPII are still 93.5% identical. The identity between the two mouse CRABPs is 74%, with the major amino acid changes found on hydrophilic residues that contribute to the surface of the proteins (Kleywegt et al., 1994). Both CRABPI and CRABPII bind all-*trans*-RA but CRABPI demonstrates a ten-fold higher affinity for this ligand (Norris et al., 1994). This difference suggests that CRABPI and CRABPII play distinct roles. While additional

ligands for the CRABPs have been identified including 4-oxo-RA, it is interesting to note that neither CRABPI or CRABPII appears to bind 9-*cis*-RA, the preferred ligand for the RXRs (Allenby et al., 1993).

The expression pattern of the CRABPs during murine embryogenesis has been well documented. Both CRABPI and CRABPII transcripts are expressed at all stages of development in distinct often non-overlapping patterns (Ruberte et al., 1992). In the adult mouse, however, CRABPII is apparently restricted to the skin (Giguere et al., 1990). CRABPI appears more ubiquitous in its expression with adult rat brain, retina, thymus, lung and reproductive organs all expressing this protein (Ong et al., 1982).

The expression, and response to RA exposure of CRABPI and CRABPII mRNA in various skin samples and cell lines has also been outlined. To date CRABPI has only been detected in cultured human melanocytes (Eller, 1992), surgical basal cell carcinoma specimens (Vahlquist et al., 1996) and F9 teratocarcinoma cells (MacGregor et al., 1992). Human epidermis biopsies and cultures of dermal fibroblasts (Si et al., 1995), lung fibroblasts (Astrom et al., 1991), and keratinocytes (Sanquer et al., 1993) do not express CRABPI mRNA constitutively. Incubation with RA did not result in induction of CRABPI mRNA in any of the cell lines studied. CRABPII is more ubiquitous in its expression. Transcripts for this gene have been observed in adult human epidermis, cultured dermal fibroblasts (Astrom et al., 1991), keratinocytes (Sanquer et al., 1993), as well as basal and squamous cell carcinomas specimens (Vahlquist et al., 1996). Transcripts were not observed in IMR 90 lung fibroblast cell lines or mammary epithelial cells (Si et al., 1995). Incubation of F9 cells and dermal fibroblasts with RA results in a rapid and sustained induction of CRABPII. In F9 cells treatment with 10^{-7} M RA results in a fifty-fold increase in CRABPII levels at 24 hours. Dermal fibroblasts also show a dose-dependent increase in CRABPII, however, maximal induction in these cells occurs at 6 hours, and by 72 hours a 5-fold decrease from the maximum is apparent. The expression of CRABPI or CRABPII by melanoma cells has not previously been reported.

The notable differences in the expression of the two CRABPs may be related to the different compositions of their promoters (MacGregor et al., 1992). Their differential responses to RA can be explained by the recent discovery that murine CRABPII possesses two RAREs that are recognized by RAR-RXR heterodimers (Durand et al., 1992).

The role that CRABPs play in cells has not been completely defined. It was originally proposed that CRABPI was involved in transporting RA to its nuclear receptors (Takase et al., 1986). However, there is conflicting data regarding the subcellular localization of this protein. Donovan et al. show nuclear localization of CRABPI in some cells with apparent exclusion from the nucleus in others (Donovan et al., 1995). It has also been postulated that CRABPI sequesters RA in the cytoplasm limiting its availability to the nuclear receptors (Maden et al., 1988). Support for this idea came from the overexpression of CRABPI in F9 cells (Boylan and Gudas, 1991). At low concentrations of RA these cells exhibit an 80-90% reduction in their expression of known RA-sensitive genes including RAR β_2 and laminin B1. At higher concentrations of RA this reduction is eliminated. CRABPI is also implicated directly and indirectly in the catabolism of RA (Napoli, 1996). Fiorella et al. demonstrate that CRABPI-RA complexes are substrates for microsomal enzymes involved in their conversion to more polar metabolites (Fiorella and Napoli, 1991). This is supported by the observation that an increase in polar metabolites was observed in F9 cells engineered to overexpress CRABPI (Boylan and Gudas, 1991).

Despite the high degree of evolutionary conservation manifest in these proteins they do not appear to be required for normal murine embryogenesis or adult life. Mutant mice were generated that lacked either CRABPI (Gorry et al., 1994), CRABPII or both CRABPI and CRABPII (Lampron et al., 1995). With the exception of minor limb abnormalities they were essentially normal and, as embryos, not more sensitive to RA administration than their wild-type littermates. A primary function for the CRABPs in maintaining intracellular RA levels during periods of reduced Vitamin A intake is now being examined by the authors.

STATEMENT OF PURPOSE

In studies of invasion and metastasis the underlying objective is to uncover basic biological mechanisms that will eventually allow the development of rational approaches to therapy. This thesis examines if the therapeutic efficacy of RA can be correlated to alterations in the production of extracellular-matrix-degrading proteases, the cellular retinoic acid-binding proteins or the nuclear receptors for RA. Two well characterized murine melanoma cell lines of differing metastatic capacity are examined.

MATERIALS AND METHODS

CELL CULTURE

Murine melanoma cell lines of differing metastatic capacity, B16-F1 low capacity and B16-F10 high capacity (Poste et al., 1980), were obtained from the American Type Culture Collection. Cells were grown in monolayer cultures at 37^{o} C in 5% CO_2- 95% air. The cells were maintained with Dulbecco's modified Eagle's media (DMEM), 10% fetal bovine serum (FBS), 50 U/ul penicillin and 50 ug/ml streptomycin. At confluence the lines were serially passaged using trypsin-EDTA to detach the cells.

RETINOIC ACID

All-trans retinoic acid (RA)was obtained from Sigma. A stock solution of 10^{-2} M was made by dissolving the appropriate quantity in ethanol, and stored at -20^{o} C protected from light. Stock solutions were diluted with DMEM to the required concentrations, used once and discarded.

CELL PROLIFERATION

B16-F1 and B16-F10 cells were grown to near confluence, washed twice with phosphate buffered saline (PBS), treated with trypsin, centrifuged and resuspended in the growth media described above. After counting with a hemacytometer, 3.0 x 10^4 cells were seeded in duplicate into six well plates. Cells were treated with either retinoic-acid, at concentrations from 10^{-8} M to 10^{-5} M, or with ethanol. The final concentration of ethanol in all wells was 0.1% volume/volume. After 48 hours the cells were removed with trypsin and the numbers determined using either a hemacytometer or coulter-counter. The percentage growth inhibition of proliferation was calculated as (1-R/E) x 100 where: R = number of cells treated with retinoic-acid and E = number of cells treated with ethanol (Hendrix et al., 1990).

RNA ISOLATION

Total RNA was isolated from RA treated and control cells using the acid guanidinium thiocyanate method described by Chomczynski and Sacchi (1987). Quantitation of the resulting DNA was determined by absorbance at 260 nm and the quality assessed by absorbance at 280 nm.

Polyadenylated mRNA selection was accomplished using the polyATract mRNA isolation system kit (Promega). Briefly total cellular RNA was precipitated with 1/10 of a volume 3M NaOAC, 2 volumes 100% ethanol, washed with 70% ethanol and resuspended in DEPC treated water to 5 ug/ul. 250ug of RNA was then heated to 65^{O} C for 10 minutes. 3 ul of biotinylated oligo dT and 13 ul of 20x SSC was added and the mixture allowed to cool to room temperature. Strepavidin paramagnetic particles were washed 3 times in 0.5x SSC, resuspended in 0.1 ml of 0.5x SSC and added to the oligo dT/RNA mix for 10 minutes. Beads with bound poly(A)$^+$ mRNA were then captured with a magnetic stand using 4 washes of 0.1x SSC. RNA was eluted from the beads using one wash of 0.1 ml DEPC treated water and one wash of 0.25 ml DEPC treated water. Excess beads were

removed by centrifugation for 5 minutes at 14,000 g at 4° C. Quantitation and purity of the resulting poly(A)$^+$ RNA was assessed as described above.

NORTHERN BLOTS

RNA samples containing either 25 ug of total cellular RNA or 2.5 ug of poly(A)$^+$ RNA were resuspended in 5 ul of DEPC treated water and 20 ul of sample buffer (20mM MOPS, 5mM NaOAC, 1mM EDTA, 7.4% formaldehyde, 50% formamide and 0.05 mg/ml ethidium bromide). Samples were then heated to 70° C for 10 minutes and briefly placed on ice. 2 ul of 50% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol was added to each sample.

The RNA was size fractionated on a 1% agarose gel containing 3.7% formaldehyde and 1x salt stocks (20mM MOPS, 5mM NaOAC and 1mM EDTA) in a running buffer of 1x salt stocks. Capillary transfer to Hybond N membrane filters (Amersham) was carried out overnight in 10x SSC. After transfer the membranes were photographed and RNA immobilization achieved by UV cross-linking at 0.2 Joules/cm^2.

The membranes were prehybridized at 42° C for one hour in 6x SSC, 5x Denhardt's solution, 50% formamide, 5% SDS and 100 ug/ml salmon sperm DNA. cDNA probes were labeled with ^{32}P to a specific activity of approximately 1x 10^6 cpm/ml (as described below) and denatured by boiling for 10 minutes. The membranes were then hybridized overnight at 42° C in 6x SSC, 50% formamide, 0.5% SDS and 100 ug/ml salmon sperm DNA. Blots were initially washed twice at room temperature for 15 minutes in 2x SSC, 0.1% SDS and then washed twice at 42° C for 15 minutes in 0.2x SSC, 0.1% SDS. Autoradiography was performed for 2-7 days at -80° C with Kodak film and intensifying screens. Band sizes were determined by comparison to a Gibco BRL RNA ladder. Membranes to be reprobed were stripped by boiling in 0.1% SDS and allowing to cool to room temperature. The membranes were subjected to autoradiography to verify probe removal.

COMPLEMENTARY DNA (cDNA) PROBES

The cDNA for murine CRABPI was present in a pJB20 vector (constructed by L. Roman) and released by EcoR1 digestion. The resulting full length cDNA included 74 base pairs of 5' untranslated DNA and the 3' polyadenylation signal sequence (Zhang, 1991). The cDNA for murine CRABPII was the gift of Dr. Giguere (University of Toronto, Canada). A full length, 863 base pair fragment resulted from the EcoR1 digestion of pBluescript IIKS.

The cDNA for human RAR α, RAR β and RAR γ were the gift of Dr. Chambon (Strasbourg, France). RAR α cDNA was the result of EcoR1 digestion of PSG5 (Petkovich et al., 1987). RAR β cDNA was obtained by the Sac1-BamH1 digestion of PSG5 (Brand et al., 1988) and RAR γ cDNA was obtained by the EcoR1 digestion of PSG5 (Krust et al., 1989).

A full length, 1864 base pair fragment containing the entire coding region of chicken β-actin resulted from Pst1 digestion of PGEM3z. All cDNA fragments were gel purified following manufacturer's recommendations (Geneclean Inc.) and then radioactively labeled following the protocol developed by Feinberg and Vogelstein (Feinberg and Vogelstein, 1983). Briefly, the DNA was denatured at 100^{o} C for 10 minutes and cooled on ice. A mixture of hexanucleotides, dATP, dGTP, dCTP, [α ^{32}P] dCTP and Klenow was added. The reaction was incubated at 37^{o} C for 30 minutes and stopped by the addition of EDTA (pH 8.0). Unincorporated nucleotides were removed using a Nunc Push Trap column (Stratagene).

NON-DENATURING WESTERN BLOTS

PROTEIN ISOLATION: RA and control treated cells were detached using trypsin, washed twice with PBS and lysed in 50mM Tris (pH 7.5), 25mM NaCl, 2.5mM EDTA, 1mM DTT, 1ug/ml leupeptin, antipain and pepstatin, 10uM benzamidine and 2uM

phenylmethylsulfonyl fluoride (PMSF). The solution was then put on ice for 20 minutes. In order to decrease viscosity the solution was passed through a syringe with a 20 gauge needle three times and centrifuged for 20 minutes at 10,000 g. The final protein concentration was determined using a protein assay kit (BioRad) with bovine serum albumin (Sigma) serving as the standard.

100 ug of each sample was loaded in a total volume of 25 ul with 5 ul of sample buffer (final concentration 0.01% bromphenol blue, 4.5% glycerol). Samples were subjected to electrophoresis in 7.5% non-denaturing gels at 40 mA. Non-denaturing gels were run in order to separate CRABPI and CRABPII according to their differing isoelectric points (5.0 and 5.46 respectively) (Siegenthaler, 1990). This allowed simultaneous detection on one gel of both proteins through use of the R851 antibody generated by J. Zhang (Zhang, 1991). By western analysis it has been previously demonstrated that antibody R851 can recognize both CRABPI and CRABPII under these conditions (Gillen and Roman, (1996, submitted)).

Proteins were transferred onto 0.2uM nitrocellulose membranes (Schleicher and Schuell) in a Biorad electrophoretic transfer apparatus containing 195mM glycine and 25mM Tris base. After transfer, membranes were stained with Ponceau-S (Sigma) to ensure efficient transfer and equal loading. Non-specific binding sites were blocked for at least 2 hours with 5% non-fat dry milk in TBS (0.1M Tris, 0.15M NaCl, 0.07% Tween-20) and 0.02% sodium azide. After the membranes were washed 4x with TBS they were incubated at a 1:2500 dilution of 1^{o} antibody R851 for 1 hour and then washed as before. 2^{o} antibody, goat anti-rabbit IgG (H and L) horseradish peroxidase (IgG-HRP, Gibco BRL) was diluted 1:2500 and also incubated for 1 hour. Two further TBS washes followed by one TBS wash containing 0.5M NaCl were performed. Immunocomplexes were detected using an electrochemiluminescence kit (Amersham).

tPA ASSAY

tPA activity from conditioned media was assayed with a tPA/PAI kit (American Diagnostica). This assay employs the chromogenic substrate methylsulfonyl-D-cyclohexyltyrosyl-glycyl-arginine-p-nitroaniline acetate. Detection of free p-nitroaniline generated by tPA cleavage is determined spectrophotometrically at an OD of 405nm. Briefly 100 ul of 1M acetic acid (pH 3.9) was added to duplicate samples (200 ul each) of media. Control samples consisted of media with no cells grown in it. All samples were stored at -80^{o} C until assayed. Samples were thawed to room temperature, centrifuged at 4,000g for 5 minutes and 100 ul of the supernatant was assayed according to kit instructions. A calibration curve consisting of known International Units (IU) of tPA was generated to allow for determination of absolute tPA levels. Values were normalized to account for total cell protein calculated using a Biorad kit.

IN VITRO TRANSCRIPTION

In vitro transcription of CRABPI and CRAPBII cDNA was performed to provide positive controls for Northern blots. A transcription kit (Promega) was used and the manufacturers instructions followed.

RESULTS

CELL PROLIFERATION

B16-F1 and B16-F10 cells were treated with retinoic acid for 48 hours and the effects on proliferation examined. RA treatment resulted in a clear inhibition of growth in both cell lines (Table 1). This inhibition was dose-dependent over concentrations of RA from 10^{-8} M to 10^{-5} M. The B16-F1 subline appeared to be more sensitive to the inhibition in growth than the highly metastatic B16-F10 line (Fig. 2). At 10^{-8} M RA, B16-F1 growth was inhibited by 17% while there was no appreciable effect on the growth of the B16-F10

cells. At higher concentrations of RA (10^{-5} M) this difference disappeared with both cell lines showing approximately 45% less growth. A direct cytotoxic effect of RA at this concentration was ruled out through careful examination of the tissues culture plates prior to counting for non-viable cells.

tPA ACTIVITY

Plasminogen activators have been implicated as playing a key role in tumor cell invasion through BMs (Mignatti and Rifkin, 1993), however, overexpression of these enzymes has also been linked to decreased tumor cell invasion (Tsuboi and Rifkin, 1990). tPA activity following RA treatment was measured indirectly using a chromogenic assay that reflects the production of plasmin as described in Materials and Methods. B16-F1 and B16-F10 cell lines were found to secrete equal amounts of tPA at baseline, and treatment with RA for 48 hours resulted in increased secretion by both cell lines (Fig 3). This increase was dose-dependent for RA concentrations of 10^{-8} M to 10^{-6} M. B16 F10 cells doubled their plasmin generation from 23.6 IU/ml to 47.5 IU/ml while B16-F1 cells tripled their production from 18 IU/ml to 53.3 IU/ml. No significant differences were seen between the two cell lines in tPA activity following RA treatment.

NUCLEAR RAR mRNA INDUCTION

The expression of mRNA for the nuclear RARs was analyzed using northern blot hybridization. Approximately 2.5ug of poly(A)+ selected mRNA was run in each lane. Equal loading was assured by reprobing the blot with an actin probe. Figure 4. shows that RAR α is expressed as two transcripts of approximately 3.2kb and 2.4kb which is consistent with previous reports (Clifford et al., 1990). Expression of RAR α occurs in both sublines with higher levels detected in the B16-F10 cell line. As in many other cell types, treatment of these cell lines with RA does not alter the expression of RAR α.

Effects of RA on murine melanoma cell proliferation

Treatment	Number of cells (x 10^5)		
	B16 F1	B16 F10	p value
Ethanol	4.20 +/- 0.35	4.01 +/- 0.30	0.374
10^{-8} M	3.50 +/- 0.36	4.21 +/- 0.42	0.016
10^{-7} M	3.25 +/- 0.36	3.83 +/- 0.67	0.075
10^{-6} M	2.64 +/- 0.29	3.05 +/- 0.21	0.071
10^{-5} M	2.40 +/- 0.43	2.08 +/- 0.11	0.135

Table 1. Cells were plated at 3 x 10^4 cells/well in DMEM supplemented with 10% FBS. After attaching for 12 hours they were washed and incubated for 48 hours in either the appropriate concentrations of RA (dissolved in ethanol) or 0.1% ethanol. Values represent the means +/- the standard deviations of duplicate samples in two separate experiments. Statistical analysis was performed using a a paired Student's t-test.

Percentage growth inhibition by RA

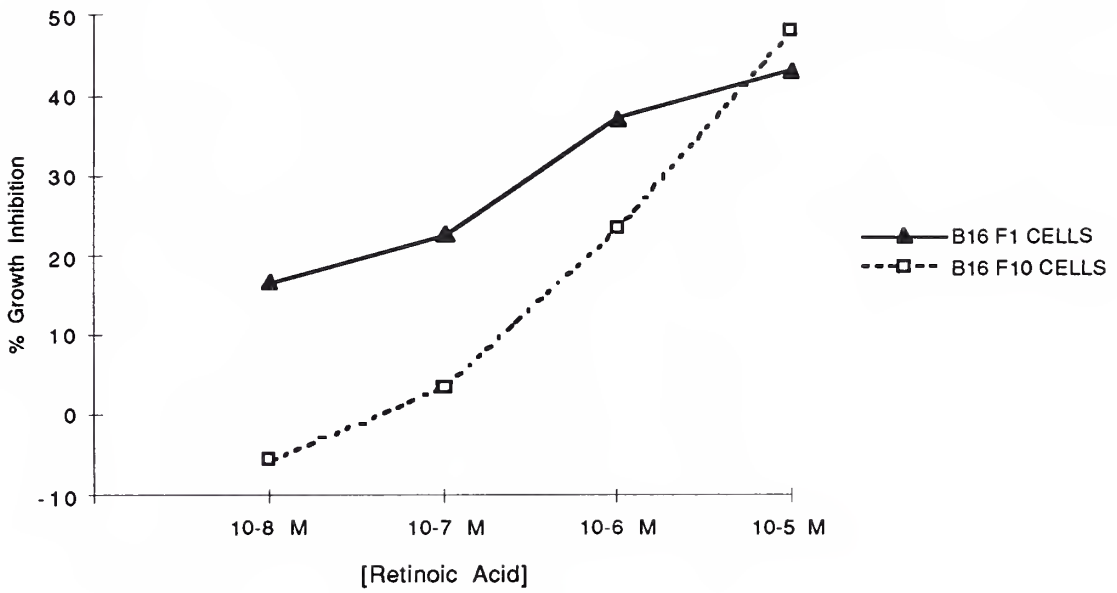

Figure 2. Percentage growth inhibition was derived from the data in Table 1and represents the results of duplicate samples from two separate experiments . The formula (1 - RA/E) x 100 was employed where: RA = number of cells counted after treatment with retinoic acid and E = number of cells counted after treatment with ethanol.

Effects of RA on murine melanoma cell tPA activity

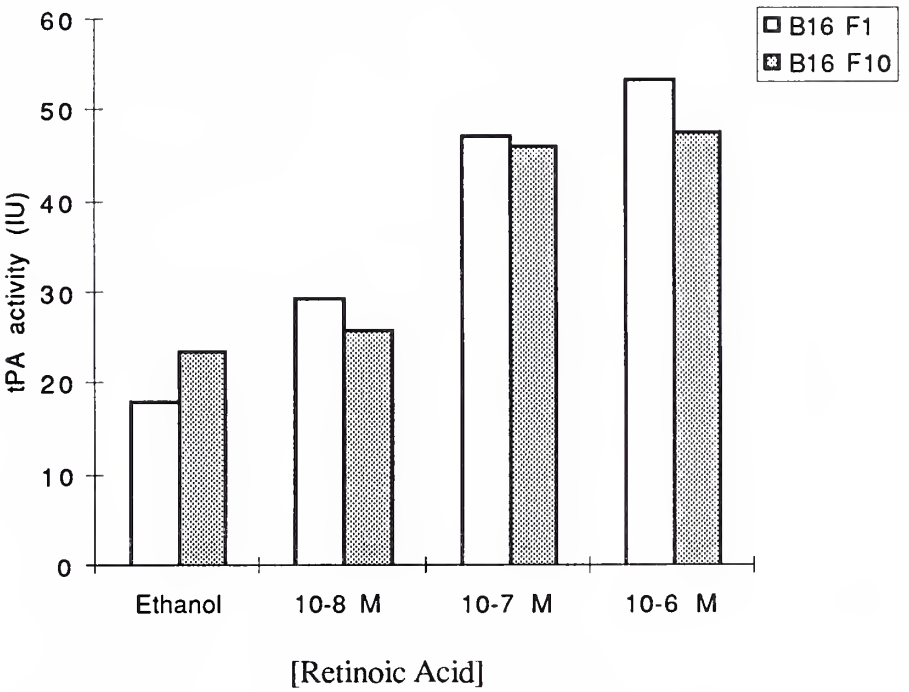

Figure 3. Cells were treated with either retinoic acid (dissolved in ethanol) or 0.1% ethanol for 48 hours. tPA activity in the conditioned media was determined with a chromogenic assay as described in the Materials and methods. Data shown represent the results of duplicate samples from one experiment. Statistical analysis using Student's t-test did not reveal significant differences between B16-F1 and B16-F10 cell lines.

The RAR β gene is known to have a RARE, and we were interested in examining its induction by RA. The middle panel of figure 4. shows that RAR β transcripts were not detectable in untreated cells. A definite dose-dependent induction of RAR β mRNA was noted in B16-F10 cells after treatment with RA. A similar but less impressive induction of RAR β transcription was also seen in B16-F1 cells.

Although RAR γ is reported to be constitutively expressed in B16-F1 cells (Niles, 1995) and (Clifford et al., 1990) its transcription was not detected in either cell line before or after incubation with RA.

CRABPI AND CRABPII PROTEIN INDUCTION BY RA

Use of the R851 antibody allowed detection of both CRABPI and CRABPII by non-denaturing western blot analysis as described in the Materials and Methods section. Figure 5. demonstrates that neither B16-F1 nor B16-F10 cell lines produce detectable levels of CRABPI , and that levels of this protein do not appear to be changed following treatment of cells with retinoic acid for 72 hours. Similarly baseline expression of CRABPII does not occur in either cell line. There is however, a dose-dependent increase in CRAPBII expression in B16-F10 cells following RA treatment. This expression is evident at concentrations of RA as low as 10^{-8} M and continues through concentrations of 10^{-6} M. In contrast, B16-F1 cells do not alter their production of CRABPII in response to RA. The positive control in this blot is an F9 cell line transfected to overexpress CRABPI (Gillen, 1996).

CRABPI and CRABPII mRNA RESPONSE TO RA

We used northern blot analysis to examine if RA was inducing changes in the mRNA levels of the CRABPs that were not being reflected in the production of a stable protein. The top panel of Figure 6 demonstrates that 72 hour incubation with RA

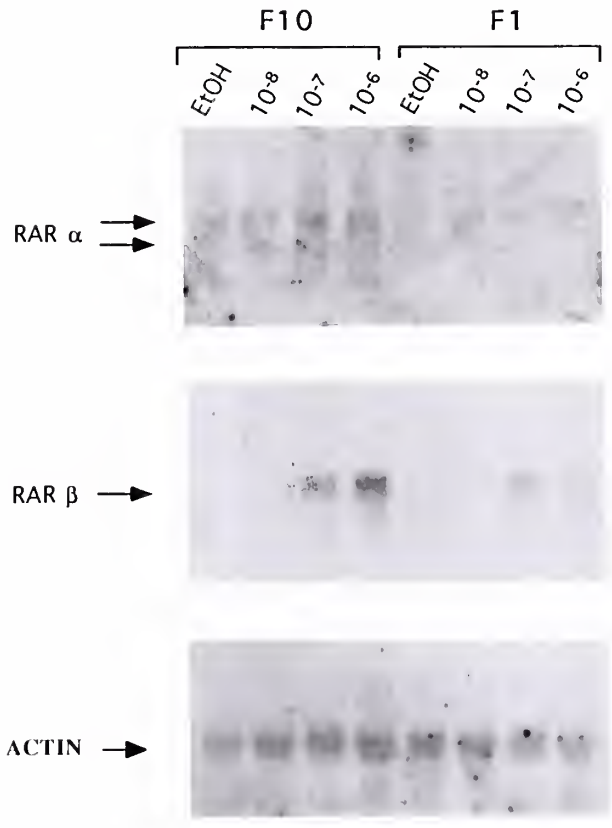

Figure 4. Northern analysis of RAR α and RAR β mRNA induction.

B16 F1 and F10 cells were seeded at equal numbers in 100mm dishes overnight. The following day cells were treated with either 0.1% ethanol or RA at various concentration for 48 hours. 2.5ug of poly(A)$^+$ selected mRNA was subjected to formaldehyde gel electrophoresis and transferred to a nylon membrane. The blot was first hybridized with a cDNA probe for RAR β and analyzed by autoradiography. The same blot was then stripped and the hybridization repeated with a β-actin probe followed by a RAR α probe.

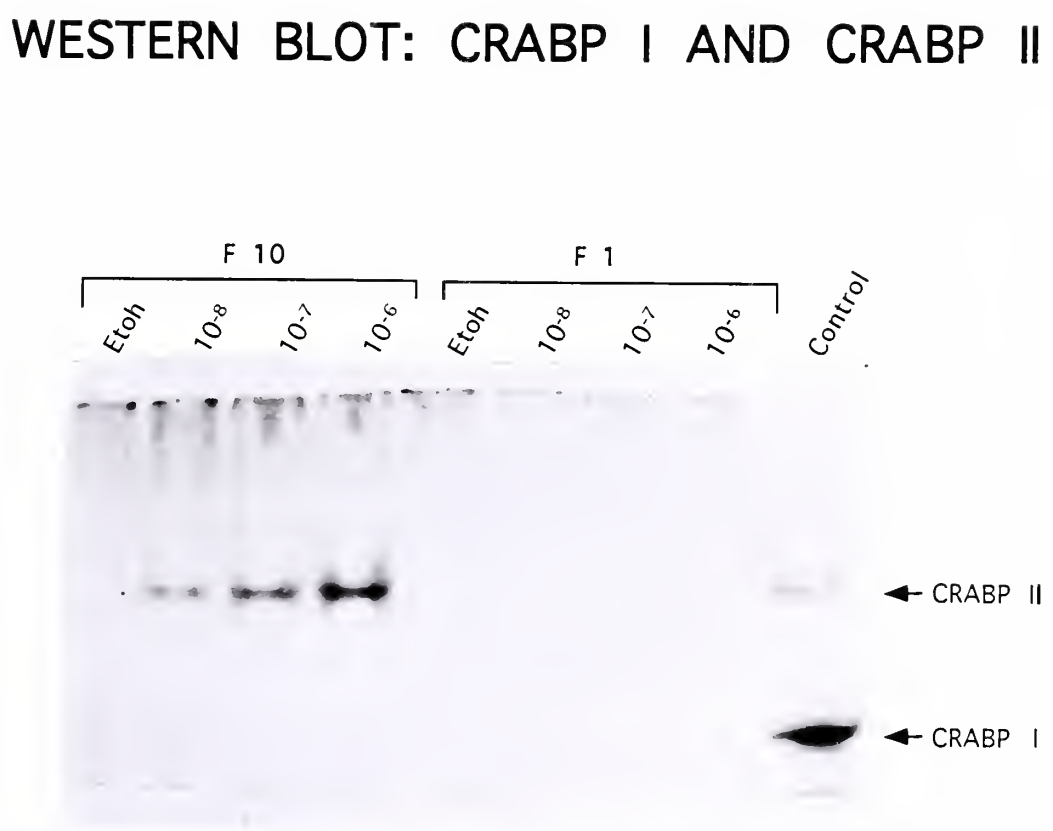

Figure 5. Western analysis of CRABPI and CRABPII protein induction.

Cells were incubated with RA as described for northern analysis, and protein isolation carried out as outlined in the Materials and Methods section. 100ug of either RA or ethanol treated sample was loaded in each lane, subjected to electrophoresis in a non-denaturing gel and transferred to nitrocellulose membrane. 1° antibody R851 was applied at a 1:2500 dilution followed by anti-rabbit IgG-HRP. Immunocomplexes were detected using electrochemiluminescence. Control lane contains 75ug of protein from a F9 cell line transfected to overexpress CRABPI.

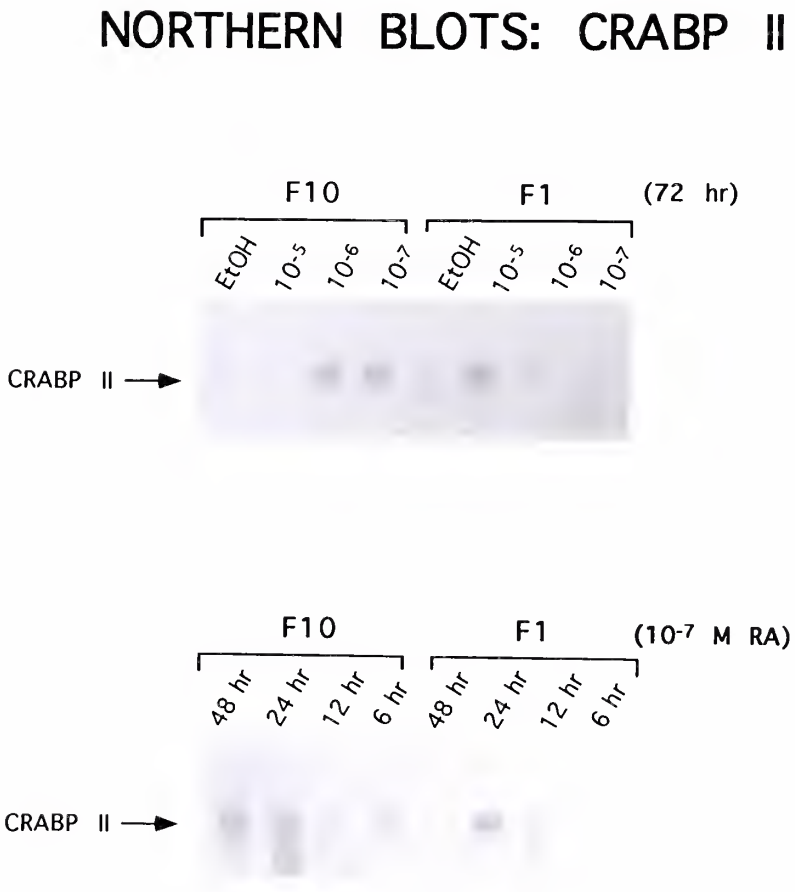

Figure 6. RA concentration-dependence and time course studies of CRABPII expression.

B16 F1 and F10 cells were seeded at equal numbers in 100mm dishes overnight. The following day cells were treated as described below. 2.5ug of poly(A)$^+$ selected mRNA was then subjected to formaldehyde gel electrophoresis and transferred to a nylon membrane. Both blots were hybridized with a cDNA probe for CRABPII and analyzed by autoradiography. Upper panel: Cells were treated with either 0.1% ethanol or RA at various concentrations for 72 hours. Lower panel: Cells were treated with 10^{-7} M RA for various periods of time.

concentrations of 10^{-7} M and 10^{-6} M can induce transcription of CRABPII in B16-F10 cells. Higher concentrations of RA (10^{-5} M) are required before transcription is effected in B16-F1 cells. As it has been reported in F9 cells that the murine CRABPII gene is maximally expressed only after 24 hours in the presence of RA (MacGregor et al., 1992) we carried out a time course of its induction. The lower panel of Figure 6 shows a rapid induction within 6 hours of CRABPII in B16-F10 cells and that high levels of transcripts are still detectable at 48 hours. The B16-F1 cell line showed induction occurring later at 12 hours, reaching maximal levels at 24 hours and returning to baseline levels by 48 hours.

CRABPI is neither expressed nor inducible by RA in B16-F10 and B16-F1 cells (data not shown).

DISCUSSION

RA is known to have a broad range of effects in decreasing tumor cell invasion and metastasis. This thesis was an attempt to analyze the effects of RA on two murine melanoma cell lines of differing metastatic capacities. Recent work by Supino et al., (1994) on two human melanoma clones with similar basal characteristics, revealed marked differences in plasminogen activation and invasion through a reconstituted BM following RA treatment . We investigated whether B16 sublines also demonstrated heterogenous responses to RA, and if these responses could be correlated to alterations in RA binding proteins.

This study showed a clear inhibition of growth in both cell lines over RA concentrations ranging from 10^{-8} M to 10^{-5} M. The B16-F1 cell line was more sensitive to these effects. These observation confirm previously reported work (Sartorelli, 1981). The levels of inhibition obtained for F10 cells are nearly identical to the reported study, however, the anti-proliferative effects of RA on FI cells they observed were twice as high

as seen in our study. B16 melanoma sublines are known to contain heterogenous cell populations (Poste et al., 1980) and the differences in these results may be explained by the selection of slightly altered populations for testing. The exact mechanism by which RA inhibits melanoma cell proliferation is unknown but may be mediated by the expression of its binding proteins (Si et al., 1996).

Plasminogen activators play a key role in tumor invasion. PAs are serine proteases that convert plasminogen to plasmin, a protease able to activate numerous pro-enzymes and degrade multiple components of the ECM. In B16 melanoma cells the total level of PA activity is correlated with invasion (Wang et al., 1980), however, the relative contributions of uPA and tPA to this process are not clear. Our data indicate that the highly metastatic F10 cells and the less metastatic F1 cells secrete equal amounts of tPA at baseline. While this is in direct contrast to reports that untreated F1 cells have higher levels of tPA in their conditioned media (Huijzer et al., 1995), differences in methodology (a direct chromogenic assay versus zymography) may account for this discrepancy. In addition, repetition of this experiment would be necessary before any definitive claims regarding tPA secretion are made. A direct correlation between tPA secretion and invasiveness however, appears unlikely. The dose-dependent increase in tPA secretion by both clones following RA exposure is somewhat surprising since it has been reported that human melanoma sublines treated with RA attenuate the expression of this enzyme and show inhibited invasion through reconstituted BMs (Hendrix et al., 1990). The overexpression of tPA by murine melanoma cells could also serve to decrease their invasive potential. The indiscriminate destruction of extracellular-matrix components may interrupt interactions required for cell anchorage and locomotion. Further characterization of these cell lines with regard to plasmin inhibitors and uPA activity may reveal heterogeneous responses to RA not seen with indirect assays of secreted tPA.

Since nuclear receptors are the likely mediators of retinoid-induced changes in gene expression we used northern blot analysis to examine the levels of RAR α, β and γ. We

show that RAR α is constitutively expressed, while RAR β is induced by RA concentrations of 10^{-7} M and 10^{-6} M in both cell lines. These findings have been reported for B16-F1 cells (Xiao et al., 1996) but are new for B16-F10 cells. In contrast to Xiao et al., we were unable to detect RAR γ mRNA in either cell line. This failure may have been secondary to our probe selection as the authors report that they found it necessary to use a relatively G-C poor restriction fragment for successful hybridization to RAR γ. The direct involvement of RAR β in growth inhibition in melanoma cells appears unlikely given the work of Clifford et al. These authors found that in S91-C2, B16-F1 and K1735P cells RA treatment inhibited growth but that RAR β was induced only in S91-C2 and B16-F1 cells. A further cell line S91-C154 showed RAR β induction without any growth inhibition

The cellular retinoic acid-binding proteins were next examined to determine if their levels could be correlated with the anti-proliferative effects of RA. A putative function for these proteins in binding RA and limiting its availability to nuclear receptors has been proposed (Maden et al., 1988) and we hypothesized that their overproduction by B16-F10 cells might be responsible for the decreased sensitivity of this cell line to RA. Indirect support for this hypothesis comes from two recent studies. Widschwendter et al. (1995) looked at the synergistic inhibition of proliferation by RA and interferon-alpha (IFN-α) in breast cancer cell lines. Synergistic inhibition was seen in two cell lines BT-20 and SKBR-3 but not in MC-7 cells. In MC-7 cells a marked increase in CRABPII was noted after combination treatment . In contrast in BT-20 and SKBR-3 cells combination therapy clearly reduced RA-mediated CRABPII induction. Delva et al. examined cells from patients with acute promyelocytic leukemia before and after relapse from RA therapy (Delva et al., 1993). The relapsing cells showed significantly increased levels of CRABPII and decreased responsiveness to RA administration. Previous expression of these proteins by melanoma cells is not recorded, however, human melanocytes have been shown by northern blot analysis to express only CRABPI and not CRABPII (Eller, 1992). In this study we were unable to detect CRABPI mRNA or protein either before or after RA

administration. This result may reflect species specific expression of this gene or alternatively its downregulation with conversion of cells to malignancy.

In contrast to CRABPI the expression of CRABPII is regulated by RA in a number of tissues (MacGregor et al., 1992) and (Astrom et al., 1991). Our data indicate that its induction in murine melanoma cells is also possible. Western blot analysis carried out after 48 hours of RA treatment demonstrated induction of CRABPII only in B16-F10 cells. This provides support for our hypothesis that this protein may mediated RA's anti-proliferative effect in this subline. Northern blot analysis confirmed this result and indicated that extremely high doses of RA (10^{-5} M) are necessary to induce CRABPII in B16-F1 cells. As the growth inhibitory effect of RA was noted by 48 hours we examined the time course of CRABPII induction. Increased expression of CRABPII after only 2 hours, reaching a maximum at 24 hours and sustained for 72 hours is reported in human dermal fibroblasts after exposure to RA (Si et al., 1995). Using 10^{-7} M RA we noted a similar induction in CRABPII mRNA that started by 6 hours and continued throughout a 48 hour time period. The kinetics of this RA-associated increase in CRABPII mRNA are consistent with its involvement in the regulation of cell proliferation.

SUMMARY

The effects of retinoic acid on two murine melanoma cell lines of differing metastatic potential were examined. A clear dose-dependent inhibition of proliferation was noted in both cell lines. The less metastatic F1 cells appeared more sensitive to this effect. The secretion of tPA, a pivotal protein involved in tumor invasion, was also studied. RA increased the expression of this protein by both cell lines in a dose-dependent manner. Expression of the cellular retinoic acid-binding proteins and nuclear retinoic acid receptors before and after RA exposure was determined in an attempt to correlate their levels to the observed inhibition of proliferation. Clear induction of RAR β and CRABPII was evident

with increased levels of both seen in F10 cells. A protective effect for CRABPII is proposed against RA-induced growth inhibition.

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